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The **overall hypothesis** that drives this project is that persistent replication stress generates mutational events in breast epithelial cells that fuel breast cancer (BCa) progression. Our model predicts that a major source of replicative stress in BCa is hypoxia, which stalls active replication forks, and selects for cells that have bypassed the this S-phase checkpoint due to mutations in the ATR-hChk1 pathway. The specific aims of this project are: (1) to define the role of the ATR checkpoint pathway in hypoxia-induced cytostasis, and (2) to determine whether defects in this checkpoint pathway promotes BCa progression, and confers sensitivity to killing by certain anticancer agents.

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INTRODUCTION

The identification of genome surveillance proteins, such as *BRCA1*, *BRCA2, ATM*, *hCHK1*, and *hCHK2*, as BCa susceptibility genes leads to the hypothesis that defects in S phase checkpoint function are major contributors to breast cancer development and/or progression. In a report that served as the background for this proposal, we demonstrated that deep hypoxia (0.1% O_2) caused cancer cells to arrest in S phase of the cell cycle, and that this arrest was accompanied by the activation of the ATR-hhChk1 checkpoint pathway (1). These results prompted speculation that escape from the ATR-hChk1 –induced growth arrest confers a proliferative advantage to malignant clones in the developing tumor. At the same time, these clones will gain an increased level of genomic instability due to the disruption of the ATR-dependent S phase checkpoint. Thus, hypoxia-induced S phase arrest may fuel BCa tumorigenesis in part by increasing the rate of gene mutation in the affected BCa cells. We further contend that errors incurred during DNA replication represent a major source of mutations that drive BCa development.

This project is intended to further understand the interplay between tumor hypoxia-reoxygenation, the ATR-hChk1 checkpoint pathway, and malignant progression in human BCa. Our goal is to establish relevant in vitro culture systems involving human breast epithelial cells (hBrEC) that are either precancerous or fully malignant. These model systems will be used to dissect the roles of surveillance proteins such as ATR, hChk1 and BRCA1, as suppressors of tumor progression, and the impact of defective ATR-mediated checkpoint activation on genetic instability and the emergence of fully tumorigenic BCa clones. Finally, using the knowledge gained from these studies, we will explore rational therapeutic strategies targeted against cells bearing defective ATR-dependent S phase checkpoints.

BODY

Task 1: To define the role of the ATR checkpoint pathway in hypoxia-induced cytostasis, and to determine whether hypoxic adaptation promotes genetic instability in human BCa cells (Months 1-18).

The continued pursuit of the Task 1 studies during the second year of this project has uncovered a novel mechanism that impacts not only on our understanding of the interplay between hypoxia and genetic instability in BCa, but also on the tumor responsiveness to a major class of cancer chemotherapeutic agents. We presented the initial results in last year's progress report, and the line of investigation was developed over the past year to the point that a major paper was submitted to *Cell*. We are currently revising the paper for re-submission to the same journal. I am pleased to report that this work was funded almost entirely by my BRCP award. This work should change existing paradigms regarding the relationship between the ATR-hhChk1 pathway and cancer development.

As outlined in our original application, we have been investigating the possibility that the ATR-hChk1 pathway protects cancer cells from replication stress induced by low oxygen and nutrient conditions, which are frequently observed in developing, avascular microtumors. Our rationale is that a number of tumor suppressor genes relevant to breast cancer, including BRCA1 and BRCA2, play major roles in the recovery of stalled replication forks of replication arrest induced by various genotoxic insults, including, we believe, hypoxia-reoxygenation-induced stress. Furthermore, as stated in our application, several of our most successful anticancer drugs, including camptothecin and gemcitabine, target S-phase, and we hypothesize that the therapeutic indices of these drugs stem in part from their selective effects on tumor cells that are struggling to complete S phase due to stressful environmental conditions. In last year's Progress Report, I presented evidence that hypoxia sensitizes

BCa cells to killing by clinically relevant concentrations of CPT. During the course of these studies, we made the unexpected observation that exposure of MCF-7 BCa cells to hypoxia caused the disappearance of the hChk1 kinase **(Figure 1A)**, a key target of ATR in the replication stress-response pathway (2, 3). We also observed striking decreases in hChk1 protein levels in cells exposed to genotoxic agents, including CPT, which are known to impair replication fork progression in S phase cells **(Figure 1B)**.

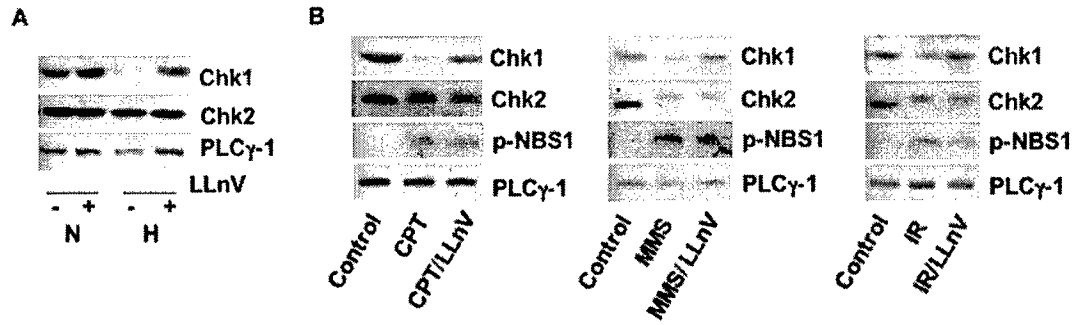

Figure 1. Hypoxia-induced Chk1 degradation. MCF-7 breast cancer cells were exposed to normoxia (20% O2) or deep hypoxia (0.1% O2) for 8 h, in the absence or presence of the proteasome inhibitor, LLnV. hChk1 protein levels were determined by immunoblotting.

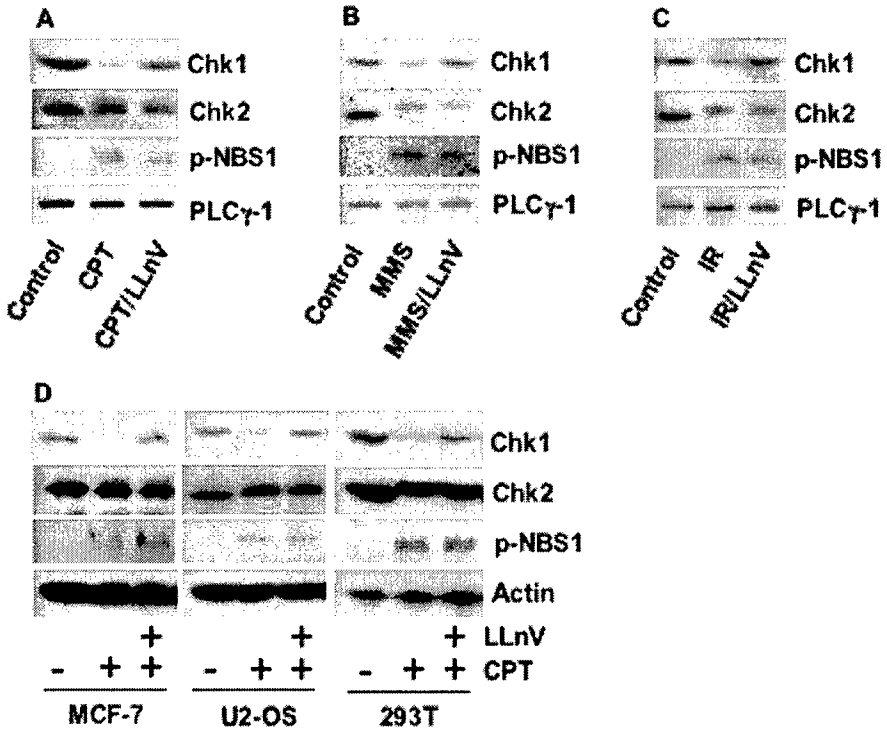

Figure 2. Proteasome-dependent Chk1 degradation. (A) A549 cells were treated for 8 h with 500 nM CPT, in the absence or presence of 2 μM LLnV during the last 4 h in culture. Cell extracts were immunoblotted with the indicated antibodies. **(B)** A549 cells were treated for 8 h with 0.05% MMS and 2 μM LLnV, and samples were analyzed as described in *panel A*. **(C)** A549 Cells were treated with 10 Gy IR, and LLnV was added at 4 h post-irradiation. Samples were analyzed after an additional 4 h. **(D)** The indicated cell lines were treated with 500 nM CPT and 2 μM LLnV as described in *panel A*. Cell extracts were immunoblotted with the indicated antibodies.

Further experimentation revealed that the loss of hChk1 in human BCa, as well as normal epithelial cell lines, was due to the degradation of the protein by the ubiquitin-proteasome pathway. Using CPT as the replication stress-inducing agent, we showed that the loss of hChk1 could be largely rescued by treatment of the cells with the proteasome inhibitor, LLnV **(Figures 1 and 2)**. Remarkably, we discovered that the signal for degradation of hChk1 was the very same signal that triggered hChk1 activation in CPT- or hypoxic cells – the phosphorylation of hChk1 at Ser-345 by the ATR kinase **(Figure 3)**. A search of the literature indicated to us that phosphorylation of several proteins, including a number of cell-cycle regulatory proteins, marked these proteins for poly-ubiquitination by a family of ubiquitin E3 ligases known as the cullin-ring ligases (CRLs) (4, 5). Seven cullin subunits

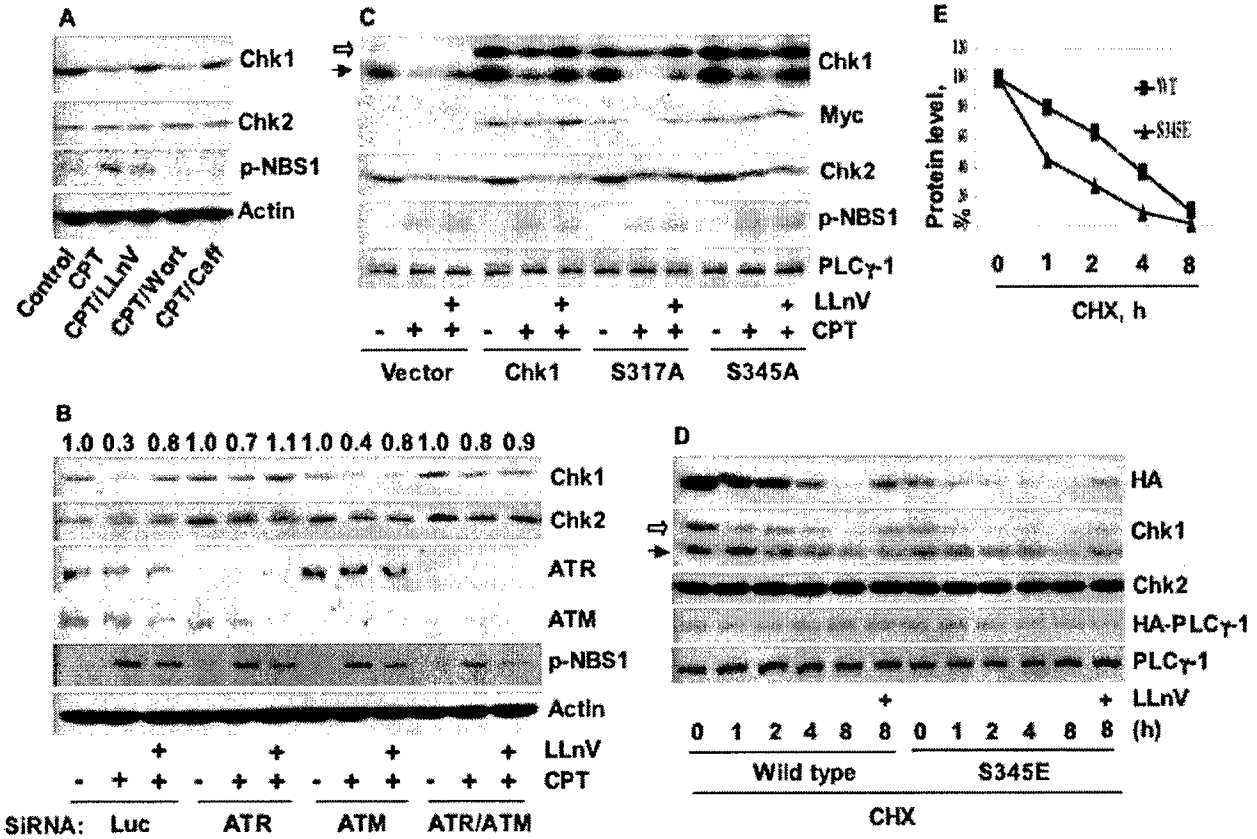

Figure 3. Role of ATR-dependent phosphorylation in Chk1 degradation. **(A)** MCF-7 cells were pretreated for 20 min with 10 μM wortmannin (wort) or 10 mM caffeine (caff), and then were cultured for 8 h in the presence of 500 nM CPT. Where indicated, LLnV was added during the last 4 h of CPT treatment. Protein expression was analyzed by immunoblotting with the indicated antibodies. **(B)** U2-OS cells were transfected with siRNAs targeted against luciferase (Luc), ATR, ATM, or ATR/ATM. After 48 h, the cells were treated for 4 h with CPT, followed by addition of LLnV to the indicated samples. After an additional 4 h in culture, the cells were lysed and proteins were immunoblotted with the indicated proteins. Numbers at the top of each sample lane represent the Chk1 protein level, normalized to that obtained in the no-drug control for each siRNA-transfected cell population. **(C)** HEK 293T cells were transfected with empty vector, or expression vectors encoding Myc-Chk1 wild type, Myc-Chk1 S317A, or Myc-Chk1 S345A proteins. After 48 h, the cells were treated for 8 h with CPT and LLnV as described in *panel B*. Protein expression was analyzed by immunoblotting. The broad and narrow arrows represent the ectopically expressed and endogenous Chk1 proteins, respectively. **(D)** HEK293T cells were co-transfected with HA-tagged Chk1 wild type or S345E expression plasmids, together with an HA-PLC-γ1 expression plasmid to monitor transfection efficiency. After 48 h, the cells were treated with 160 μM cycloheximide (CHX) for the indicated time periods. Protein expression was analyzed by immunoblotting. The broad and narrow arrows represent the ectopically expressed and endogenous Chk1 proteins, respectively. **(E)** The α-HA immunoblot in (D) was quantitated by densitometry, and sample values were normalized to the 0 h control, which was set at 100%.

are expressed in human cells. Using both overexpression and small-interfering RNA (siRNA) strategies, we examined the potential roles of these cullins in CPT-induced hChk1 degradation, and two – cullin 1 and cullin 4A – emerged as key regulators of hChk1 degradation in MCF-7 and other cancer cell types. We are currently searching for the F-box proteins that associate with cullin 1 and cullin 4A to form the E3 ligases that target the phosphorylated, activated form of hChk1 for proteolysis.

Why are these findings significant for breast cancer development and therapy? We know that Chk1 function is essential for the viability of normal cells, due to the fact that, even under the best of circumstances, replication forks are prone to stalling due to base misincorporation errors or aberrantly structured DNA (e.g., fragile sites) (6, 7). Stalled replication forks must remain viable until the initiating insult is repaired, and it turns out that Chk1 is required for replication fork viability and replication re-initiation after stalling (8). In the absence of Chk1, forks disassemble, and the host cell is unable to completely replicate its genome. Because the damage to the genome is typically massive under these conditions, cell death due to apoptosis or premature chromosome condensation is the inevitable consequence of replication fork breakdown. Our discovery that ATR-dependent phosphorylation of hChk1 both activates this kinase and marks it for degradation may seem counter-productive to the goal of preserving cell viability in the face of genotoxic stress. However, we hypothesize the replication stress pathway operates continuously during a normal S-phase, and that sub-populations of hChk1 molecules are continuously activated in order to maintain the movement of individual replication forks. Under normal circumstances, this pool of activated hChk1 must be removed shortly after it performs its essential function as a countermeasure to replicative stress. Otherwise, the accumulation of activated hChk1 molecules would likely reach a critical threshold, above which otherwise undamaged cells would be unable to complete S-phase. This coupled mechanism of activation and degradation works nicely under conditions of normal-low level replicative stress, but likely leads to a pathological loss of hChk1 after prolonged or high-intensity stress to the DNA replication machinery. The hypoxic tumor microenvironment provides one such setting, and cancer chemotherapy with agents such as CPT, 5-FU, and gemcitabine represent another situation in which extreme loss of hChk1 may lead to irreversible S-phase arrest and, ultimately cell death.

The relevance of this novel mechanism of hChk1 regulation to cancer therapy was established in dramatic fashion by the finding that this mechanism was abrogated in cell in certain drug-resistant cell lines, including the BCa line, MDA-MB-231. CPT is an effective antitumor agent, but resistance to this drug is a major problem in the clinic. Our studies demonstrate that the loss of hChk1 protein is intimately related to the cytotoxic activity of CPT, and that disruption of the Chk1 degradation pathway leads to hChk1 resistance **(Figure 4)**. The significance of this finding was documented by results indicating that knockdown of hChk1 expression with siRNA reversed the CPT resistance of MDA-MB-231 cells. Thus, this research project has not only shed new light on the antitumor mechanism of CPT, but also reveals a common pathway leading to CPT resistance, and likely resistance to other S-phase genotoxins as well.

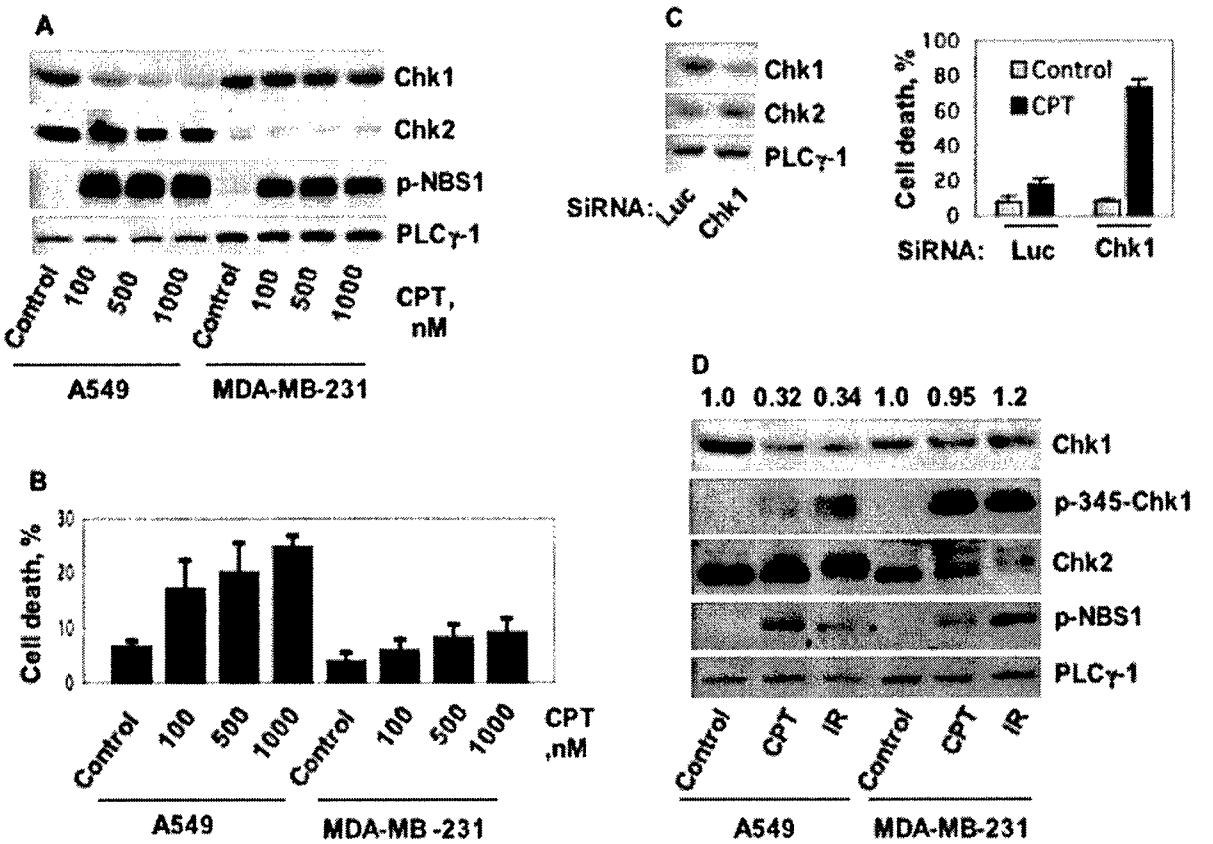

Figure 4. Chk1 expression as a determinant of CPT sensitivity. **(A)** A549 and MDA-MB-231 cells were treated for 8 h with the indicated concentrations of CPT. Protein expression levels were determined by immunoblotting with the indicated antibodies. **(B)** Cells treated as described in *panel A* were cultured for 48 h in fresh medium. Cell death was determined with the Trypan Blue dye exclusion assay. The data are plotted as mean +/- standard deviation from 3 independent trials. **(C)** MDA-MB-231 cells were transfected for 48 h with the indicated siRNAs. *Left panel,* expression levels of Chk1 and Chk2 proteins were determined by immunoblotting. *Right panel*, siRNA-transfected cells were treated for 8 h with 500 nM CPT. Drug-containing medium was replaced with fresh medium, and cells were cultured for an additional 36 h. Cell death was measured as described in *panel B*. The data are presented as mean +/- standard deviation from three independent experiments. **(D)** A549 and MDA-MB-231 cells were treated with 500 nM CPT or 20 Gy IR. After 8 h, cell extracts were prepared and immunoblotted with the indicated antibodies. Numbers at the top of each sample lane represents the Chk1 protein level, normalized to that obtained in the non-treated control for each cell population.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that both hypoxia and S-phase specific anticancer agents activate the ATR-hChk1 pathway, leading to the poly-ubiquitination and proteolytic degradation of hChk1.

- Discovered a novel mechanism whereby the activation of ATR by replication stress leads to the phosphorylation of hChk1 at Ser-345, a modification that simultaneously activates the Chk1 kinase domain and targets the protein by cullin 1- and cullin 4A-containing E3 ligases.

- Provided novel insights into the antitumor mechanism of CPT, and demonstrated that breast cancer cells acquire resistance to CPT through disruption of the hChk1 degradation pathway.

- Generated inducible hChk1 cell lines for further studies in project year 3.

REPORTABLE OUTCOMES

The major reportable outcome for project year 2 is that a manuscript describing replication stress-induced degradation of hChk1 is currently in the review process:

Zhang Y-W, Otterness DM, and Abraham RT: Genotoxic Stress Targets Human Chk1 for Degradation by the Ubiquitin-Proteasome Pathway. *Cell*, in revision, 2005.

CONCLUSIONS

During project years 1-2, we have generated the human breast cancer cell lines, and have established the technology and protocols for hypoxia-reoxygenation studies. These studies have revealed that replication stress leads to the activation of an ATR/hChk1-mediated checkpoint response that allows these cells to restart stalled replication forks after the initiating insult is resolved. Our studies also demonstrated that persistent or high-intensity stress leads to the degradation of hChk1, causing extensive DNA damage in cells exposed to hypoxia-reoxygenation. Furthermore, this hChk1 degradation response plays a key role in CPT-induced antitumor activity. Finally, we have defined a novel mechanism for CPT resistance in BCa and other cancer cell lines. In many cases, these drug-resistant cells fail to degrade hChk1 in response to CPT exposure, which allows the cells to maintain viability and resume cell proliferation after termination of CPT therapy. Our plan for year 3 is to further explore the mechanism and consequences of hChk1 degradation in BCa and other malignant cell lines, and to move our newly generated, inducible cell lines to the *in vivo* antitumor studies described in task 2 of the original application.

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